no results confirming the superiority of EGFR-TKI even showing clinical or genetic prediction of a better response, because these trials failed to show better survival over standard combination regimens including platinum agents.

We continue to await the results of research which will demonstrate clinical benefits in terms of survival even in selected patients, and which may help us to identify patients who are most likely to benefit from treatment with EGFR-TKI. Such results would tell us when and to whom we should prescribe the best drug to treat NSCLC.

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EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib

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Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumour response to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer (NSCLC). Pleural effusion is a common complication of lung cancer. In this study, we assessed the feasibility of detection of *EGFR* mutations in samples of pleural effusion fluid. We obtained 43 samples, which was the cell-free supernatant of pleural fluid, from Japanese NSCLC patients, and examined them for *EGFR* mutations. The *epidermal growth factor receptor* mutation status was determined by a direct sequencing method (exons 18–21 in *EGFR*). *EGFR* mutations were detected in 11 cases (E746_A750del in seven cases, E746_T751del insA in one case, L747_T751del in one case, and L858R in two cases). The *EGFR* mutations were observed more frequently in women and non-smokers. A comparison between the *EGFR* mutant status and the response to gefitinib in the 27 patients who received gefitinib revealed that all seven patients with partial response and one of the seven patients with stable disease had an *EGFR* mutation. No *EGFR* mutations were detected in the patients with progressive disease. The results suggest that DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status may be useful as a predictor of the response to gefitinib.

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Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (Parkin *et al*, 2005). Most patients have advanced disease at the time of diagnosis. The initial therapy for advanced non-small-cell lung cancer (NSCLC) is systemic chemotherapy with a two-drug combination regimen, which often includes a platinum agent, but the median survival of patients treated with such regimens has ranged from only 8 to 10 months. Little improvement in the efficacy of chemotherapy has been made in the last 20 years (Breathnach *et al*, 2001; Kelly *et al*, 2001; Schiller *et al*, 2002).

Targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of NSCLC, because EGFR has been found to be expressed, sometimes strongly, in NSCLC (Franklin *et al*, 2002). Gefitinib ('Iressa', AstraZeneca) is a small molecule and selective EGFR tyrosine kinase inhibitor that has shown antitumour activity in NSCLC patients as a single agent in phase II trials (Fukuoka *et al*, 2003). Adding gefitinib to chemotherapy in phase III studies of patients with untreated advanced NSCLC did not significantly improve the outcome over chemotherapy alone (Giaccone *et al*, 2004; Herbst *et al*, 2004), and a possible explanation for the failure to observe any added benefit in these trials is that the patients had not been screened or selected for their ability to derive any clinical benefit from an EGFR inhibitor.

An association between mutations in sites of *EGFR* tyrosine kinase in NSCLC and hyper-responsiveness to gefitinib has recently been reported (Lynch *et al*, 2004; Paez *et al*, 2004). The mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of *EGFR*, and the mutations increased the affinity of the enzyme for ATP and gefitinib (Lynch *et al*, 2004). Some investigators subsequently found that *EGFR* mutations are one of the strong determinants of tumour response to EGFR tyrosine kinase inhibitors (Pao *et al*, 2004; Han *et al*, 2005). The investigators

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used surgical tissue to detect the *EGFR* mutations in their studies, but most patients who require gefitinib therapy are diagnosed at an advanced stage of the disease and are inoperable. As it is often difficult to obtain a sufficient tumour sample from patients with inoperable NSCLC to detect *EGFR* mutations by direct sequencing, a method of detecting *EGFR* mutations in other specimens needed to be established.

Malignant pleural effusion is a common complication of lung cancer. It is present in approximately 15% of patients at the time of diagnosis (Pass *et al*, 2005) and in 10–50% of patients during the course of the disease (Fenton and David Richardson, 1995). In about half of NSCLC patients with a pleural effusion, the effusion fluid is cytologically positive at the first time examined, and ultimately most effusions are determined to be malignant. As pleural effusion fluid sampling is usually easy, non-invasive, and repeatable, we hypothesised that tumour-derived DNA in the pleural effusion fluid of NSCLC patients would be a source of useful information on the status of the *EGFR* gene and could allow prediction of the response to gefitinib. Some investigators have reported that pleural effusion fluid is a useful clinical specimen for searching for point mutations in oncogenes, such as *K-ras*, *rho A*, *p53*, and *FHIT* (Nakamoto *et al*, 2001; Lee *et al*, 2004). As the two trials were small, the results regarding the sensitivity and specificity of detection of the mutations in pleural effusion as a diagnostic method were unclear. Detection of *EGFR* mutations in pleural effusion fluid has been described in one case report, and the patient responded to gefitinib (Huang *et al*, 2005). The results in that patient encouraged us to hypothesise that the *EGFR* mutation status determined in pleural effusion fluid is useful for predicting the responsiveness to EGFR tyrosine kinase inhibitors.

In the present study, we attempted to detect *EGFR* mutations in pleural effusion fluid and to clarify the usefulness of their detection as a predictor of the response to gefitinib.

PATIENTS AND METHODS

Patients

The subjects were NSCLC patients who had a pleural effusion at the time of diagnosis. The diagnosis of NSCLC was based on the histological or cytological findings, and the histological type was determined according to the WHO criteria (Travis *et al*, 1999). Patients' records consisted of age, gender, smoking habit, histological type, and treatment. Smoking status was collected from the patients' records. Patients were divided into three groups according to their smoking status: never smokers (<100 cigarettes/lifetime), former smokers ($\geqslant 100$ cigarettes/lifetime, no smoking at present), and current smokers ($\geqslant 100$ cigarettes/lifetime). The response of the patients treated with gefitinib was evaluated every 4 or 8 weeks in accordance with the 'Response Evaluation Criteria in Solid Tumours (RECIST)' guidelines. (Therasse *et al*, 2000). Partial response (PR) and stable disease (SD) were confirmed by a sustained 4-week follow-up. This study was approved by the Institutional Review Board of the National Cancer Center Hospital and of Kanazawa University Hospital, and written informed consent was obtained from all participants. No research results were entered into the patient's records or released to the patient or the patient's physician.

Collection of pleural effusion fluid and DNA purification

The pleural effusion fluid was collected into heparinised tubes between 29 March 2005 and 30 January 2006. No particular collection method was used. A 2-ml sample of the fluid was centrifuged at 250 *g* for 10 min at room temperature, and the supernatant was collected and stored at −80°C until DNA extraction. DNA was extracted from 1 ml of the supernatant with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the blood and body fluid spin protocol in the manufacturer's instructions, with the following protocol modifications. The same column was used repeatedly until the whole sample had been processed. The DNA obtained was eluted in 50 μl of sterile bi-distilled buffer, and the extracted DNA was stored at −20°C until used. The amounts of DNA extracted were estimated with spectrophotometry.

Polymerase chain reaction amplification and direct sequencing

Exons 18, 19, 20, and 21 of the *EGFR* gene were amplified by polymerase chain reaction (PCR). The primers were designed based on the report by Lynch *et al* (2004). Genomic PCR of 1 μl of template DNA was performed in 25 μl volumes containing 0.75 U of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA), 2.5 μl of PCR buffer, 0.8 μM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The first PCR analyses were performed in a volume of 25 μl by 25 cycles consisting of a denaturation step at 94°C for 45 s, a primer annealing step at 58°C for 30 s, and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. Nested PCR was performed with 20 cycles under the same conditions as the first PCR. Sequencing of each sample was performed in duplicate with an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced in both sense and antisense directions. *Epidermal growth factor receptor* mutations detected in the initial round of sequencing were confirmed by subsequent rounds of independent PCR and sequencing reactions. Only specimens in which a mutation was identified in both rounds were recorded as mutation-positive. The sequences were compared with the GenBank-archived human sequence for *EGFR* (accession number: AY588246). The nucleic acid and protein coordinates used to name the mutations are based on NM_005228.3 and NP_005219.2, respectively.

Statistical analyses

This study was carried out as exploratory research for detecting *EGFR* mutations from pleural effusion fluid and clarifying the relationship between the mutation status and clinical manifestations. The number of enrolled patients was therefore not precalculated. Patient characteristics, including gender, tumour histology, and smoking habit were tabulated according to their mutation status. Fisher's exact test was used to test for associations between the presence of *EGFR* mutations and the patients' characteristics. The relationship between response to gefitinib and the mutation status was evaluated individually.

RESULTS

Patients and pleural effusion specimens

Forty-three patients were enrolled in this study (Table 1). Two hundred and sixty-two patients were seen with stage IIIB and IV at our institutions in the period of this study. Forty-three of the 262 patients were enrolled in this study. The enrolled patients were not all of the patients with pleural effusion because written informed consent was not obtained from any patients with pleural effusion. Their median age was 62 years (range, 39–82 years), and there were 21 females (53.8%) and 17 never smokers (43.6%). The histological and/or cytological diagnosis was adenocarcinoma in 39 patients, and squamous cell

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Table 1 Patient characteristics and *EGFR* mutation status

	(n)	EGFR mutation (n)
No. of patients	43	11 (25.6%)
Age (years)		
Median	63	
Range	39–82	
Gender		
Male	22	4 (18.2%)
Female	21	7 (33.3%)
Smoking habit		
Current	9	2 (22.2%)
Former	16	2 (12.5%)
Never	18	7 (38.9%)
Histology		
Adenocarcinoma	39	11 (28.2%)
Squamous cell carcinoma	1	0 (0%)
Large cell carcinoma	1	0 (0%)
Unclassified	2	0 (0%)
No. of patients treated with gefitinib	27	8 (29.6%)
PR	7	7 (14.3%)
SD	7	1 (0%)
PD	13	0 (0%)

EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; SD = stable disease.

carcinoma and large cell carcinoma in one each, and unclassified NSCLC in two patients. Non-small-cell lung cancer cells in the pleural effusion samples of 40 of the patients were identified cytologically. There were no malignant cells in the pleural effusion fluid of the other three patients. We have no data of the proportion of malignant cells and normal cells. Twenty-seven patients were treated with gefitinib (250 mg day^{-1}) and evaluated for a response. Eight of the 27 patients were treated with gefitinib as an initial treatment and the other 19 patients were treated with the agent as a second or third line. The others were treated with systematic chemotherapy, including a platinum agent. The results of the evaluation showed that seven of the 27 patients who received gefitinib therapy had a PR and seven had SD. The other 13 patients had progressive disease (PD). DNA was extracted from all 43 samples of pleural effusion fluid. Amounts of the DNA extracted were detectable from 27 samples at a concentration up to 144.0 ng ml^{-1}. Amounts from 16 samples were under the detectable limit.

Detection of *EGFR* mutations in pleural effusion fluid

Direct sequencing of PCR products in exons 18–21 of *EGFR* in the pleural effusion fluid of all patients allowed their mutation status to be determined. Heterozygous mutations were identified in 11 (25.6%) of the 43 patients (Table 1). Nine mutations were deletional mutations located in exon 19 (E746_A750del in seven, L746_T751del insA in one, L747_T751del in one), and two were substitution mutations located in exon 21 (L858R) (Table 2) (Figure 1). No mutations were detected in exon 18 or 20. The E746_A750 deletion and L858R substitution mutations were the most common (9 out of 11 mutations. 81.8%) and are well-known hotspot mutations described previously (Kosaka *et al*, 2004; Pao *et al*, 2004). No more than one mutation was identified per patient, and no EGFR mutations were detected in pleural effusion fluid that did not contain malignant cells.

Table 2 Site of mutations in exons 18–21 of *EGFR*

Nucleotide changes	Amino-acid changes	No. of patients
2481_2495del	E746_A750del	6
2482_2496del	E746_A750del	1
2483_2497del	E746_T753del insA	1
2486_2500del	L747_T751del	1
2819T>G	L858R	2

EGFR = epidermal growth factor receptor; del = deletion; ins = insertion. The numbering of the mutation sites was based on NM_005228.3 (nucleotide) and NP_005219.2 (amino acid).

Epidermal growth factor receptor mutation status and patients' characteristics

EGFR mutations were detected more frequently in the samples from females (7 out of 21, 33.3% of females, 4 out of 18, 22.2% of males; $P = 0.310$) and non-smokers (7 out of 17, 41.1% of non-smokers, 4 out of 22, 18.1% of current or former smokers; $P = 0.156$), although the differences were not statistically significant (Table 3). Of the 11 mutations, 63.6% were in women and 63.6% were in non-smokers. All of the patients with mutations had adenocarcinoma. No *EGFR* mutations were found in any of the patients with squamous carcinoma or large cell carcinoma. A comparison between the *EGFR* mutant status and the response to gefitinib showed that all seven patients with a PR and one of the seven patients with SD had an *EGFR* mutation. No *EGFR* mutations were detected in any of the patients with PD (Table 4). We have no response data from the 16 patients who had never treated with gefitinib, and we have not evaluated the relationship between the response to chemotherapy and the EGFR mutation status in pleural effusion fluid.

DISCUSSION

This is the first report of an analysis of the *EGFR* mutation status in DNA obtained from the pleural effusion fluid of a series of NSCLC patients and evaluation of the relationship between the mutation status and the clinical response to gefitinib. It is interesting that all patients who achieved a PR to gefitinib had the *EGFR* mutations. We hypothesised that the mutation status in DNA extracted from pleural effusion fluid would allow prediction of the clinical outcome of gefitinib therapy in NSCLC patients, and we therefore expected the pleural effusion fluid to be a practical source of DNA for detection of *EGFR* mutations. The sites of *EGFR* mutations found in this study are identical to these reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The main mutations found were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. No patients had more than one mutation. It was possible to determine the mutation status of *EGFR* by using the DNA in only 1.0 ml of pleural effusion fluid, even though the concentration of the extracted DNA specimens was in most cases below the concentration detectable by spectrophotometry (data not shown). The results of the comparison between the mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small sub-groups of NSCLC patients, such as females and never smokers, although the differences were not statistically significant. It is well known that *EGFR* mutations are frequently observed in adenocarcinomas. As 36 of the 39 patients (92.3%) enrolled in this study had adenocarcinoma, we could not evaluate differences in the frequency of the *EGFR* mutations according to the histological type. Pleural effusion occurs in lung carcinoma of all cell types, but

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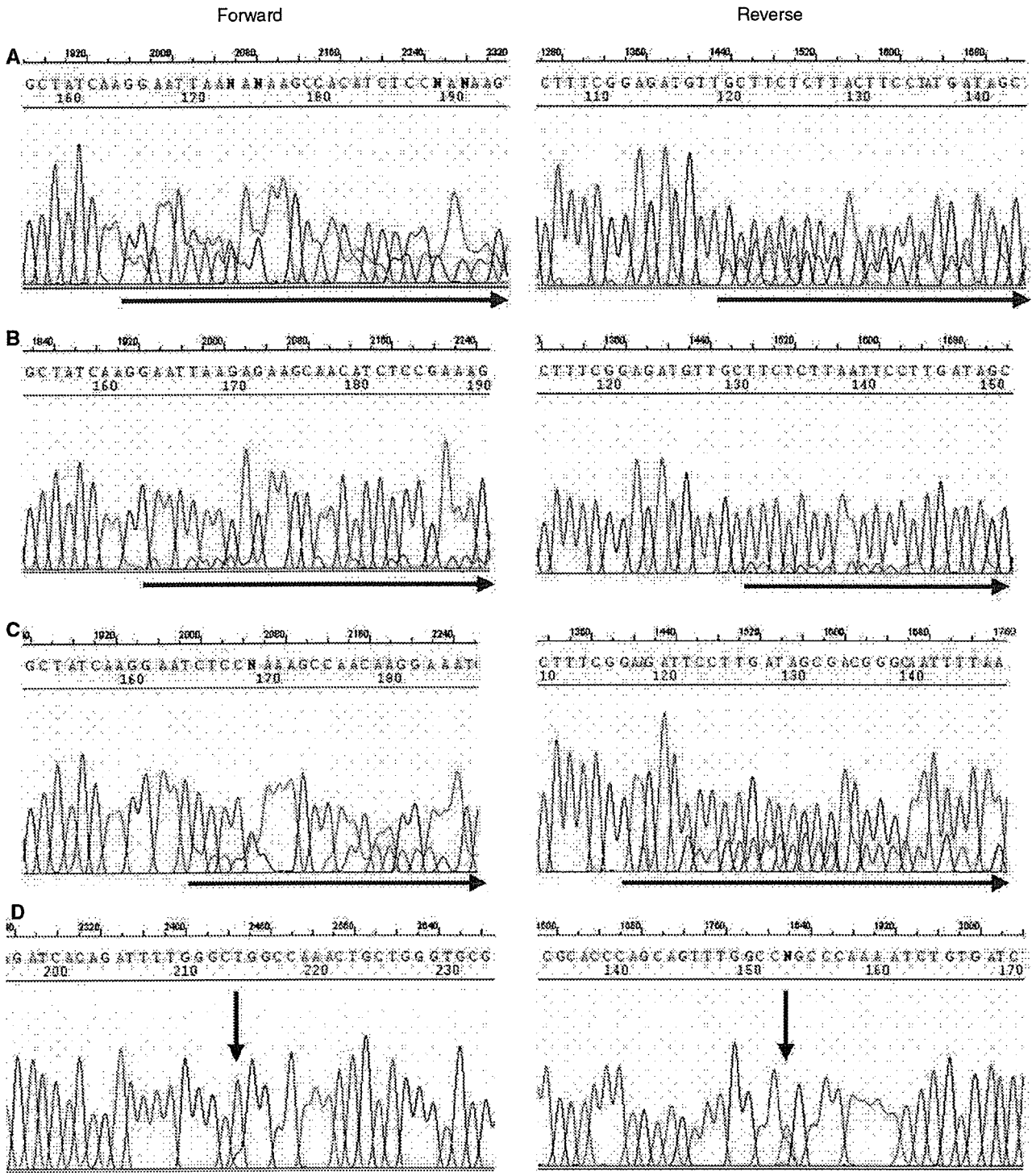

Figure 1 The wave figures of the nucleotide sequence of the *EGFR* gene with heterozygous mutations obtained by direct sequencing (see 'Patients and Methods') are shown. Horizontal arrows in both the sense and the antisense directions are shown to demonstrate the two breakpoints of the deletion. The patients in **A**, **B**, and **C** have inframe deletions in exon 19 (Figure **A**, E746_A750del; **B**, E746_T753del insA; **C**, L747_T751del; **D**, L858R). The double peaks (vertical arrows) represent the heterozygous missense mutations resulting in an amino acid substitution of L858R in exon 19 (Figure **D**).

appears to be most frequent in adenocarcinoma (Chernow and Shahn, 1997).

This study had several limitations. First, we could not compare the results of the *EGFR* mutation status in the pleural effusion fluid to the mutation status in tumour tissue. Forty of the 43 patients enrolled were cytologically diagnosed as having NSCLC from pleural effusion fluid specimens. As the DNA extracted from pleural effusion fluid consisted of DNA derived from both tumour cells and normal cells, the *EGFR* mutation status needs to be evaluated in a pair of DNA specimens from the tumour and pleural

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Table 3 Frequency of *EGFR* mutations in DNA from the pleural effusion fluid of NSCLC patients according to (A) gender, (B) histology, (C) smoking habit, and (D) response to gefitinib

(A) Gender and *EGFR* mutation status

	EGFR mutation		
	+	−	
Female	7	14	
Male	4	18	$P=0.310$

(B) Histology and *EGFR* mutation status

	EGFR mutation		
	+	−	
Ad	11	28	
Non-Ad	0	4	$P=0.558$

(C) Smoking habit and *EGFR* mutation status

	EGFR mutation		
	+	−	
Never	7	11	
Current/former	4	21	$P=0.156$

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; + = mutation-positive; − = no mutations. (A)(B)(C); a total of 43 samples were evaluated.

Table 4 *EGFR* mutation status in patients who received gefitinib therapy

Age (years)	Gender	Smoking	Histology	EGFR mutation status	Response to gefitinib
62	F	Never	Ad	E747_P753insS	PR
58	F	Never	Ad	E746_A750del	PR
80	F	Never	Ad	E746_A750del	PR
61	M	Never	Ad	E746_A750del	PR
65	M	Former	Ad	E746_A750del	PR
60	M	Current	Ad	E746_A750del	PR
66	F	Never	Ad	E747_T750del	PR
76	F	Never	Ad	Wild	SD
57	F	Former	Ad	Wild	SD
40	F	Never	Ad	Wild	SD
72	F	Never	Ad	Wild	SD
58	F	Former	Ad	Wild	SD
66	F	Never	Ad	Wild	SD
65	F	Former	Ad	L858R	SD
39	F	Never	Ad	Wild	PD
69	M	Former	Ad	Wild	PD
72	F	Never	Ad	Wild	PD
74	F	Never	Ad	Wild	PD
67	M	Former	Ad	Wild	PD
62	M	Former	SCC	Wild	PD
59	F	Current	Ad	Wild	PD
77	M	Current	Ad	Wild	PD
82	F	Never	Ad	Wild	PD
66	F	Never	Ad	Wild	PD
56	M	Current	Ad	Wild	PD
61	M	Former	Ad	Wild	PD
65	M	Former	Ad	Wild	PD

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; F = female; M = male; NSCLC = unclassified NSCLC; PD = progressive disease; PR = partial response; SCC = squamous cell carcinoma; SD = stable disease.

effusion fluid to confirm the usefulness of the mutation status determined from pleural effusion fluid. However, it is sometimes difficult to obtain tumour samples from patients with advanced NSCLC, and even more so from patients diagnosed as having NSCLC using methods other than the histological examination of tumour tissue, such as on the basis of pleural effusion or sputum cytology. Second, direct sequencing may be not able to provide satisfactory results for detection of *EGFR* mutations in mixed samples of mutated and wild DNA. Although direct sequencing has generally been used to detect *EGFR* mutations in previous studies, detection of a mutation by this method requires at least 30% of the mutated DNA in a sample (Bosari *et al*, 1995; Fan *et al*, 2001). Lung cancers are very heterogeneous, and as normal cells, such as inflammatory cells or mesothelial cells, are contained in the pleural effusion fluid of lung cancer patients, in addition to tumour cells, a small amount of mutated DNA in pleural effusion fluid can be missed by direct sequencing. Unfortunately, we have no data at the present time on whether EGFR mutations were detectable in pleural effusion samples with either a few malignant cells, a small proportion of malignant cells with normal mesothelial cells, or cytologically negative samples. To establish a method for the detection of EGFR mutations from pleural effusion fluid, the mutation detectable proportion of malignant cells to normal cells in pleural fluid should be elucidated. We are planning an additional study using cytological examination to clarify the mutation detectable proportion as a next step. When pleural fluid is used as the material for detection of *EGFR* mutations, a patient with an *EGFR* mutation may be diagnosed as having wild-type *EGFR* because of the two limitations described above. Although we expected a high frequency of detection of *EGFR* mutations in this study because of the high proportion of adenocarcinomas (92.3%), we detected *EGFR* mutations in only 28.2% of the patients enrolled, a lower frequency than in two previous reports on Japanese NSCLC patients (Takano *et al*, 2005; Asano *et al*, 2006). Patients with false-negative results, meaning that no *EGFR* mutations were detected in a patient with an *EGFR* mutation, were not excluded from this study. Some investigators have tried to improve the sensitivity of detection of *EGFR* mutations in samples containing a mixture of tumour and normal cells. Wookey *et al* (2005) reported findings that the ARMS method was superior to the direct sequencing method and WAVE method for detecting *EGFR* mutations. Other groups have reported that LightCycler PCR assay (Sasaki *et al*, 2005), SSCP assay (Marchetti *et al*, 2005), and enriched PCR assay (Asano *et al*) are more sensitive than direct sequencing and are more rapid. A standardised method of detecting *EGFR* mutations needs to be established as soon as possible.

The final limitation in the present study is that it remains unclear whether there is any survival benefits associated with gefitinib therapy in those patients enrolled with *EGFR* mutations. The relationship between the *EGFR* mutation status determined in pleural effusion fluid and the gefitinib response in a portion of the patients enrolled supports the pleural effusion fluid *EGFR* mutation status as useful for predicting the response to gefitinib. The relationship between the *EGFR* mutation status determined in the pleural effusion fluid and the gefitinib response in the remaining patients and the survival benefit of gefitinib therapy in the patients with *EGFR* mutations are currently being evaluated, and confirmation of the results is expected in the very near future.

In conclusion, our results suggest that the DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status determined may be useful as a predictive factor of response to gefitinib.

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Molecular Biomarkers

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ORIGINAL ARTICLE

Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells

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Y-box-binding protein 1 (YB-1), which is a member of the DNA-binding protein family containing a cold-shock domain, has pleiotropic functions in response to various environmental stimuli. As we previously showed that YB-1 is a global marker of multidrug resistance in ovarian cancer and other tumor types. To identify YB-1-regulated genes in ovarian cancers, we investigated the expression profile of YB-1 small-interfering RNA (siRNA)-transfected ovarian cancer cells using a high-density oligonucleotide array. YB-1 knockdown by siRNA upregulated 344 genes, including *MDR1*, *thymidylate synthetase*, *S100 calcium binding protein* and *cyclin B*, and downregulated 534 genes, including *CXCR4*, *N-myc downstream regulated gene 1*, *E-cadherin* and *phospholipase C*. Exogenous serum addition stimulated YB-1 translocation from the cytoplasm to the nucleus, and treatment with Akt inhibitors as well as Akt siRNA and integrin-linked kinase (ILK) siRNA specifically blocked YB-1 nuclear localization. Inhibition of Akt activation downregulated *CXCR4* and upregulated *MDR1* (*ABCB1*) gene expression. Administration of Akt inhibitor resulted in decrease in nuclear YB-1-positive cancer cells in a xenograft animal model. Akt activation thus regulates the nuclear translocation of YB-1, affecting the expression of drug-resistance genes and other genes associated with the malignant characteristics in ovarian cancer cells. Therefore, the Akt pathway could be a novel target of disrupting the nuclear translocation of YB-1 that has important implications for further development of therapeutic strategy against ovarian cancers.
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Keywords: Akt; microarray; ovarian carcinoma; Y-box-binding protein-1

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Introduction

The Y-box-binding protein 1 (YB-1), which is a DNA/RNA-binding protein also known as dbpB, regulates transcription, translation, DNA damage repair and other biological processes in both the nucleus and cytoplasm (Matsumoto and Wolffe, 1998; Kohno *et al.*, 2003). In the cytoplasm, YB-1 regulates mRNA stability and translational regulation (Evdokimova *et al.*, 2001; Ashizuka *et al.*, 2002; Fukuda *et al.*, 2004), while in the nucleus, it plays a pivotal role in transcriptional regulation through specific recognition of the Y-box promoter element (Ladomery and Sommerville, 1995; Kohno *et al.*, 2003). Interaction of YB-1 with its cognate Y-box-binding site (inverted CCAAT box) is promoted by cytotoxic stimuli, including actinomycin D, cisplatin, etoposide, ultraviolet (UV) and heat shock, leading to the activation of a representative ABC transporter *MDR1*/*ABCB1* and DNA topoisomerase IIα genes (Asakuno *et al.*, 1994; Furukawa *et al.*, 1998; Ohga *et al.*, 1998). YB-1 also selectively interacts with damaged DNA or RNA, and protects from cytotoxic effects following cellular exposure to cisplatin, mitomycin C, UV and oxygen radicals (Ohga *et al.*, 1996; Ise *et al.*, 1999).

Royer and co-workers were the first to report that nuclear localization of YB-1 is associated with intrinsic *MDR1* expression in human primary breast cancer (Bargou *et al.*, 1997). Immunostaining analysis of various human cancers also supported this result, and showed that nuclear expression of activated YB-1 was closely associated with the acquisition of P-glycoprotein-mediated multidrug resistance (Kuwano *et al.*, 2004). YB-1 has also been shown to induce basal and 5-fluorouracil-induced expression of the major vault protein (*MVP*/*LRP*) gene, the promoter of which contains a Y-box (Stein *et al.*, 2005). In human malignancies, vault proteins are involved in acquiring drug resistance (Mossink *et al.*, 2003). Taken together, these findings suggest that nuclear localization of YB-1 might play a key role in the acquisition of global drug resistance through transcriptional activation of relevant genes and the repair of damaged DNA (Kuwano *et al.*, 2004).

The nuclear localization of YB-1 is required for transcription and DNA repair in response to various environmental stimuli, such as adenovirus infection (Holm *et al.*, 2002), DNA-damaging agents, UV irradiation, hyperthermia (Stein *et al.*, 2001) and serum stimulation (En-Nia *et al.*, 2005). However, as a nucleocytoplasmic shuttling protein, it is important to understand which signalling molecules are involved in the translocation of YB-1 into the nucleus. Koike *et al.* (1997) first reported the possible role of protein kinase C in YB-1 nuclear translocation in cancer cells exposed to UV irradiation, and highlighted the importance of the YB-1 C-terminal region in cytoplasmic retention. Other studies have suggested the involvement of additional molecules: thrombin-mediated YB-1 nuclear translocation was shown to be inhibited by protein tyrosine phosphatase inhibitor in endothelial cells (Stenina *et al.*, 2000), while Dooley *et al.* (2006) demonstrated the involvement of Jak1 in YB-1 nuclear translocation. Sutherland *et al.* (2005) recently reported that phosphorylation of YB-1 by Akt at serine 102 in the cold-shock domain is required for YB-1 nuclear translocation in cancer cells. Another mechanism for nuclear translocation of YB-1 was shown to be promoted by various cytotoxic anticancer agents, which trigger the proteolytic cleavage by the 20S proteasome of the YB-1 C-terminal fragment containing the cytoplasmic retention signal (Sorokin *et al.*, 2005). In our present study, we have provided evidence that Akt activation is one of the mechanisms for nuclear translocalization of YB-1, and also that YB-1 regulates expression of various cell growth and malignant progression-related genes as well as global drug resistance-related genes including *MDR1*.

Results

Suppression of YB-1 leads to an enhancement of MDR-1 expression and decrease of CXCR-4 expression

We previously reported that YB-1 was expressed in the nucleus in almost 30% of serous ovarian cancers, and that YB-1 nuclear-positive patients had a poor prognosis (Kamura *et al.*, 1999). As nuclear translocation of YB-1 is highly susceptible to environmental stimuli, we first examined whether the stress-inducing exogenous addition of serum could stimulate nuclear translocation of YB-1 in seven serum-deprived human ovarian cancer cell lines. Among the seven cell lines, nuclear YB-1 translocation was stimulated more than twofold in two: RMG-III and SKOV-3 (Figure 1a). In these two lines, serum incubation markedly enhanced Akt phosphorylation and increased translocation of YB-1 into the

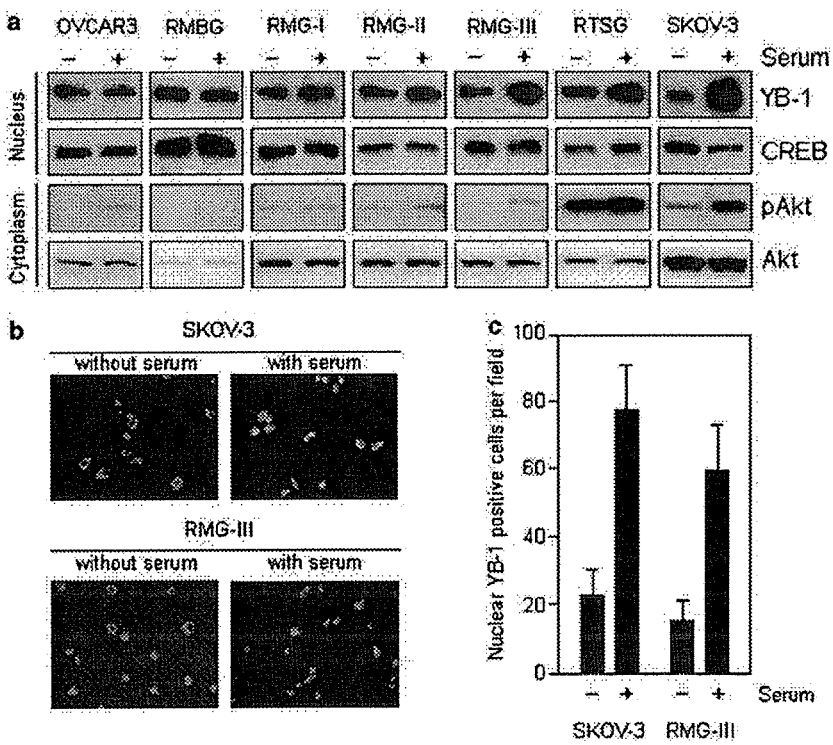

Figure 1 Levels of Akt phosphorylation and nuclear localization of YB-1 in ovarian cancer cell lines with or without serum stimulation. (**a**) Cytoplasmic and nuclear extracts were prepared 1 h after 10% serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed on nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed with cytoplasmic extracts. CREB and Akt are shown as a loading control. (**b**) Immunofluorescent staining of YB-1 in ovarian cancer cells. Cells stimulated with or without serum for 1 h were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (**c**) Quantitative analysis of YB-1 nuclear localization as shown in Figure 1b. Data are mean of three independent experiments; bars ±s.d.

nucleus, as shown by immunofluorescense analysis (Figure 1b and c).

Although YB-1 is known to regulate the expression of several genes at the transcriptional level, the complete network of genes associated with YB-1 has not been elucidated. We therefore, explored the expression profile of YB-1 siRNA-treated SKOV-3 cells and mock-treated SKOV-3 cells using a high-density oligonucleotide microarray. We transfectd YB-1 siRNA into SKOV-3 cells at a concentration of 200 and 400 nM. Transfection of 200 nM YB-1 siRNA decreased expression of YB-1 mRNA by only 45%, whereas 400 nM YB-1 siRNA decreased by 70% (Figure 2). Of the 54675 RNA transcripts and variants in the microarray, we identified 344 genes that were increased more than twofold and 534 genes that were decreased 0.5-fold or less in both 200 and 400 nM YB-1 siRNA-transfected cells (Supplementary Table S1). Upregulated genes were classified into 'cell cycle' ($P<0.0001$), 'cytoskeleton organization and biogenesis' ($P=0.0003$), 'cell growth and/or maintenance' ($P=0.0005$), and GO SLIMS Biological Process' ($P=0.0013$). Downregulated genes were classified into 'catalytic activity' ($P=0.0007$) and 'transferase' ($P=0.0010$). We selected 46 genes that we expected to be associated with drug resistance, cell growth, cancer malignant progression and cell signalling (Table 1), and chose three of these for further study: *MDR1*, *MVP/LRP* and chemokine (C-X-C motif) receptor 4 (*CXCR4*).

We used quantitative real-time PCR (QRT-PCR) to confirm whether expression of these three genes was modulated in YB-1 siRNA-transfected cells. Expression of *CXCR4* decreased by 67%, whereas expression of *MVP/LRP* was unaffected by the siRNA (Figure 2). *MDR1* expression was increased approximately 30-fold in 400 nM YB-1 siRNA-transfected cells compared with control siRNA-transfected cells. The results of QRT-PCR are broadly consistent with those of the microarray analysis.

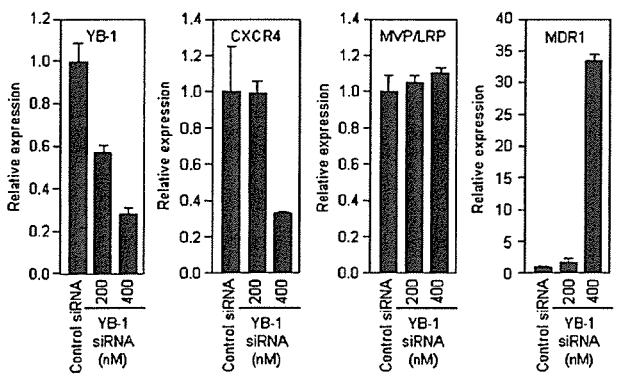

Figure 2 Effect of YB-1 knock down on expression of MDR1, MVP/LRP and CXCR4. SKOV-3 cells were treated with YB-1 siRNA for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\mathrm{GAPDH}) - C_t(\mathrm{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ± s.d.

Pearson correlation and hierarchical cluster analysis of selected NCI-60 genes

We next examined a database containing the expression profile of the National Cancer Institute (NCI)-60 panel from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), shown as a log of mRNA expression level in the NCI screen. When the Pearson correlation coefficients were calculated, YB-1 was negatively correlated with *MDR1* expression, positively correlated with *CXCR4* expression and showed little correlation with *MVP/LRP* (Figure 3). Moreover, the hierarchical dendrogram of gene expression revealed that *YB-1* and *CXCR4* belong to the same cluster, whereas *MDR1* and *MVP/LRP* are clustered in a separate group (Figure 4). Together, these NCI-60 panels suggest that cellular levels of YB-1 negatively modulate expression of *MDR1* and positively regulate expression of *CXCR4*. In this cluster analysis, six ovarian cancer cell lines including SKOV-3 showed various correlation coefficiencies with YB-1 expression. Our oligonucleotide array analysis was performed only with SKOV-3, and correlation coefficiencies among ovarian cancer cell lines would depend upon which cell line was analysed.

Akt activity is prerequisite for nuclear translocation of YB-1and transcriptional regulation by YB-1

Phosphorylation of YB-1 by Akt is a necessary requirement for its translocation from the cytoplasm into the nucleus (Sutherland *et al.*, 2005). We therefore investigated the effect of two inhibitors of Akt activation (LY294002 and 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) on serum-stimulated SKOV-3 cells. Both Akt inhibitors markedly blocked the nuclear accumulation of YB-1, whereas treatment with inhibitors of MEK (U0126), p38MAPK (SB203580) and JNK (SP600125) had no effect on nuclear translocation (Figure 5a). In addition, phosphorylation of Akt was inhibited by LY294002 and octadecylcarbonate, but not by U0126, SB203580 and SP600125. Immunofluorescence analysis with a YB-1 antibody also demonstrated the predominant accumulation of YB-1 in the cytoplasm when treated with LY294002 and octadecylcarbonate (Figure 5b and c). As Akt inhibitors blocked the nuclear translocation of YB-1, we examined whether they could also affect expression of YB-1-regulated genes. *CXCR4* expression was found to be downregulated in a dose-dependent manner following treatment with the Akt inhibitors when determined by QRT-PCR analysis (Figure 5d). Treatment with Akt inhibitors upregulated the expression of *MDR1*, but not *MVP/LRP*.

SKOV-3 cells expressed high level of Akt1 protein, very low level of Akt2 protein, and no Akt3 protein when assayed by immunoblotting analysis (Figure 6a). We introduced siRNA targeting Akt or ILK into SKOV-3 cells at a concentration of 100 and 10 nM,

Table 1 List of genes differentially expressed in YB-1 siRNA-transfected SKOV-3 cells

Unigene	*Accession*	*Symbol*	*Description*	*Mean fold change*
Hs.489033	NM_000927	ABCB1	MDR1, ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.46
Hs.369762	AB077208	TYMS	Thymidylate synthetase	1.71
Hs.198363	NM_018518	MCM10	MCM10 minichromosome maintenance deficient 10	1.70
Hs.405958	U77949	CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	1.66
Hs.442658	AB011446	AURKB	Aurora kinase B	1.65
Hs.516484	NM_005978	S100A2	S100 calcium-binding protein A2	1.48
Hs.23960	NM_031966	CCNB1	Cyclin B1	1.40
Hs.460184	AA604621	MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	1.40
Hs.438720	AF279900	MCM7	MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*)	1.36
Hs.433168	NM_002960	S100A3	S100 calcium binding protein A3	1.33
Hs.115474	NM_002915	RFC3	Replication factor C (activator 1) 3, 38 kDa	1.28
Hs.122908	NM_030928	CDT1	DNA replication factor	1.28
Hs.329989	NM_005030	PLK1	Polo-like kinase 1 (*Drosophila*)	1.21
Hs.334562	NM_001786	CDC2	Cell division cycle 2, G1 to S and G2 to M	1.21
Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22 kDa	1.19
Hs.477481	NM_004526	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	1.16
Hs.284244	M27968	FGF2	Fibroblast growth factor 2 (basic)	1.10
Hs.179565	NM_002388	MCM3	MCM3 minichromosome maintenance deficient 3 (*S. cerevisiae*)	1.08
Hs.194698	NM_004701	CCNB2	Cyclin B2	1.04
Hs.506989	BC001866	RFC5	Replication factor C (activator 1) 5, 36.5 kDa	1.02
Hs.171596	NM_004431	EPHA2	EPH receptor A2	1.01
Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	0.75
Hs.156346	NM_001067	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	0.64
Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	0.54
Hs.391464	NM_004996	ABCC1	MRP-1, ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.20
Hs.256301	NM_199249	MGC13170	Multidrug resistance-related protein	0.15
Hs.513488	NM_017458	MVP	Major vault protein	−0.05
Hs.482526	NM_014886	TINP1	TGF beta-inducible nuclear protein 1	−0.23
Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member	−1.01
Hs.500466	BG403361	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	−1.05
Hs.25292	NM_002229	JUNB	Jun B proto-oncogene	−1.06
Hs.132225	AI934473	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	−1.16
Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	−1.22
Hs.508999	NM_002742	PRKCM	Protein kinase C, mu	−1.29
Hs.326035	NM_001964	EGR1	Early growth response 1	−1.29
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	−1.32
Hs.197922	NM_018584	CaMKIINalpha	Calcium/calmodulin-dependent protein kinase II	−1.36
Hs.132966	AA005141	MET	Met proto-oncogene (hepatocyte growth factor receptor)	−1.39
Hs.208124	NM_000125	ESR1	Estrogen receptor 1	−1.50
Hs.73793	M27281	VEGF	Vascular endothelial growth factor	−1.53
Hs.381167	AW512196	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	−1.70
Hs.413111	NM_002661	PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	−1.75
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	−1.92
Hs.472793	AI631895	SGK2	Serum/glucocorticoid regulated kinase 2	−2.04
Hs.372914	NM_006096	NDRG1	*N-myc* downstream regulated gene 1	−2.34
Hs.421986	NM_001008540	CXCR4	Chemokine (C-X-C motif) receptor 4	−2.64

High-density oligonucleotide array was performed on 400 nM YB-1 siRNA-treated SKOV-3 cells and mock-treated cells. siRNA duplexes were transfected using LipofectAMINE2000 with Opti-MEM mediums. At 48 h after siRNA transfection, total RNA was prepared, and subjected to double-stranded cDNA synthesis and *in vitro* transcription. The labeled cRNA was applied to the oligonucleotide microarray.

respectively, and silencing effects of siRNA were analysed by immunoblotting (Figure 6a). In Akt siRNA almost completely silenced both Akt1 and Akt2, and siRNA for ILK, the upstream kinase for Akt, silenced ILK on protein level. Treatment with Akt siRNA and ILK siRNA resulted in a marked decrease in both pAkt expression and nuclear accumulation of YB-1 (Figure 6a). As both Akt and ILK siRNA blocked the nuclear translocation of YB-1, we examined their effects on expression of YB-1-regulated genes (Figure 6b). Treatment with Akt and ILK siRNA downregulated the expression of *CXCR4* gene, and upregulated the expression of *MDR1* gene. By contrast there appeared no marked effect on the expression of *MVP/LRP* and *YB-1* genes when treated with both siRNAs (Figure 6b).

Effect of LY294002 treatment on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft

To further investigate the involvement of Akt in tumoural YB-1 nuclear localization, an *in vivo* xenograft

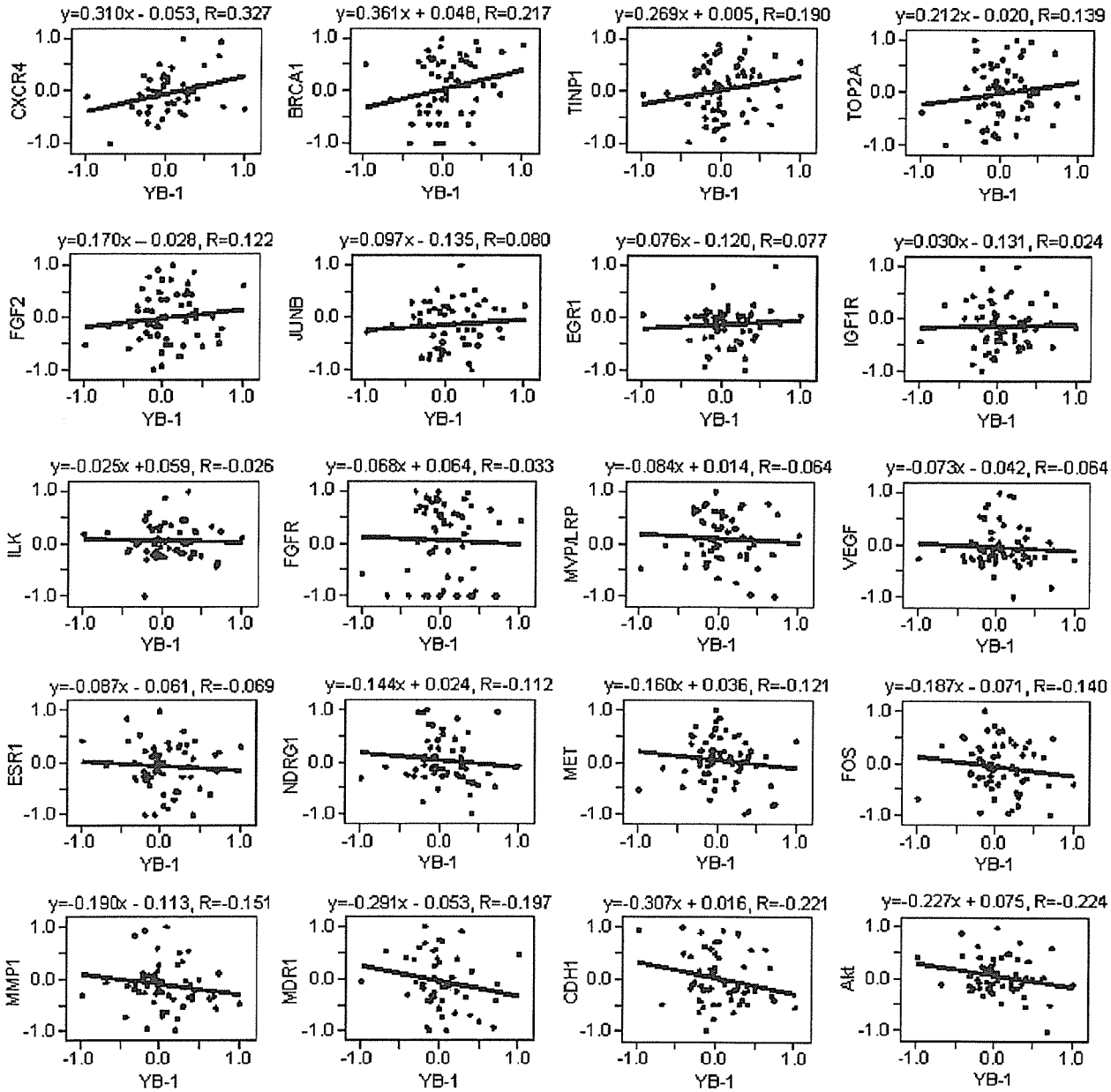

Figure 3 Correlation analysis of gene expression in NCI-60 screen. Gene expression data for the 60 human tumor cell lines were obtained from the Developmental Therapeutics Program (http://www.dtp.nci.nih.gov/), expressed as log of the mRNA levels in cell line/mRNA levels in reference pool in the NCI screen. Pearson correlation coefficients were calculated for each gene–gene pair.

assay was performed. Administration of LY294002 (i.p.) to mice carrying SKOV-3 cell tumors inhibited the phosphorylation of Akt (Figure 7a and b). Akt phosphorylation and YB-1 nuclear localization were also evaluated by immunohistochemical analysis. Tumors in the LY294002-treated group displayed a lower level of pAkt staining (3.3 ± 0.5) than those in the control group, where the mean number of nuclear YB-1-positive cells was 24.7 ± 3.4 (Figure 7c and d). Taken together, these results suggest that nuclear localization of YB-1 in ovarian cancer cells is closely associated with Akt phosphorylation activity *in vitro* and *in vivo*.

Discussion

The nuclear localization of YB-1 is essential process for YB-1-driven transcription of various genes and DNA repair in cancer cells in response to various environmental stimuli. One should understand which signalling pathway specifically controls the translocation of YB-1 from cytoplasm into nucleus. Our previous study has demonstrated that PKC activates the nuclear localization of YB-1 in cancer cells treated with UV irradiation or cisplatin, and also that the C-terminal region of YB-1 was important for its cytoplasmic

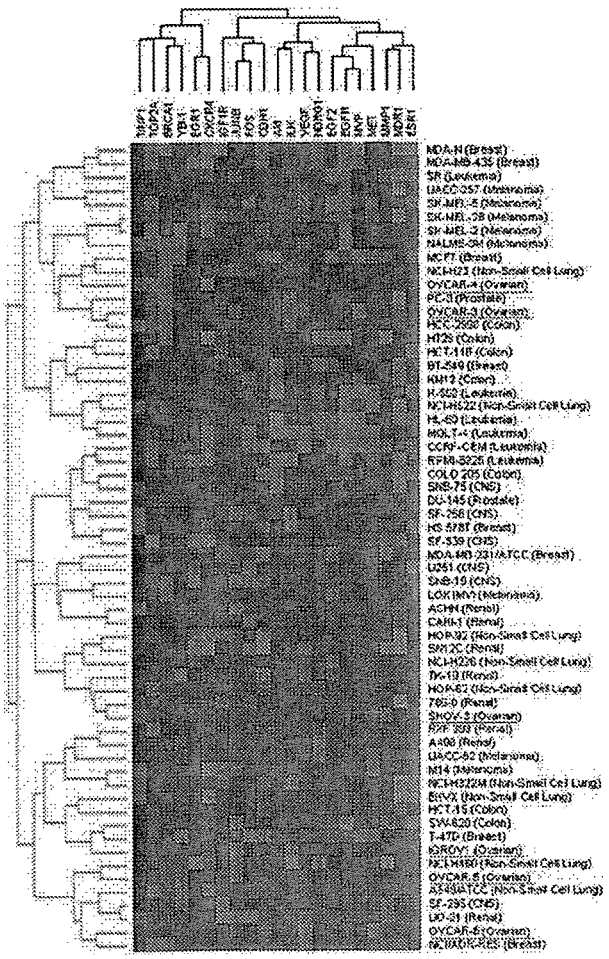

Figure 4 Hierarchical clustering of gene expression in NCI-60 screen. Hierarchical clustering can be used to group cell lines and genes in term of their patterns of gene expression. To obtain cluster trees for genes that showed distinct expression patterns across the 60 cell lines, we used the program 'Cluster' and 'Tree View' (http://rana.lbl.gov/) with average linkage clustering and a correlation metric.

retention (Koike *et al.*, 1997). Sutherland *et al.* (2005) have presented more definitive mechanism at molecular basis that phosphorylation of serine 102 at cold-shock domain of YB-1 by Akt is essential for the nuclear YB-1 localization in breast cancer cells, and also that ILK phosphorylate its downstream Akt, resulting in activation of YB-1 and it nuclear localization. Consistent with this study, our present study also demonstrated that Akt as well as ILK played a critical role in the nuclear YB-1 localization and YB-1-driven-transcriptional control of various genes including *CXCR4* and *MDR1* in human ovarian cancer cells.

In our present study, we examined whether expression of two multidrug resistance relevant genes, *MVP/LRP* and *MDR1/ABCB1*, was affected by knockdown of YB-1. Stein *et al.* (2005) have reported that the *MVP/LRP* gene is transcriptionally activated by YB-1 in response to cytotoxic anticancer agents including doxorubicin and 5-fluorouracil: *MVP/LRP* is an essential vault protein involving acquirement of multidrug resistance. However, in ovarian cancer cells, there was no causative association between the two genes when assayed by microarray and QRT-PCR. YB-1 might not regulate *MVP/LRP* expression in ovarian cancer cells used in our present study. In contrast, in human breast cancer cells, treatment with YB-1 siRNA markedly upregulated *MVP/LRP* expression (Shimoyama T, Nishio K, Basaki Y, Ono M and Kuwano M, unpublished data), suggesting that YB-1-induced regulation of *MVP/LRP* gene expression depends upon cancer cell types and/or types of stimuli. In contrast, knockdown or nuclear translocation inhibition of YB-1 upregulated expression of another drug resistance *MDR1* gene in ovarian cancer cells. Various environmental stimuli often upregulated *MDR1* gene in various human cancer cells through pleiotropic transcriptional regulations (Kuwano *et al.*, 2004). Our present study further presented a novel regulation of YB-1-induced negative control of *MDR1* gene in ovarian cancer cells, and further study should be required to understand its underlying mechanism at molecular basis.

In our present study, we first observed that the knockdown of YB-1, ILK and Akt as well as an Akt inhibitor all downregulated expression of *CXCR4* gene. Consistent with recent study by Sutherland *et al.* (2005), ILK-Akt activation could be responsible for the nuclear localization of YB-1, resulting in enhanced expression of *CXCR4* gene. The 2.6 Kb 5′-flanking region located upstream of the *CXCR4* gene contains a TATA box and the transcription start site characteristic of a functional promoter (Caruz *et al.*, 1998) and this region also contained putative consensus Y-box-binding site (inverted CCAAT box) form −685 to −681. However, it remains unknown whether ILK-Akt-induced activation of YB-1 is directly involved in the upregulation of *CXCR4* gene.

CXCL12 (SDF-1α) is a specific ligand of CXCR4. CXCL12 induced a dose dependent proliferation of human ovarian cancer cells through its specific interaction with CXCR4 (Porcile *et al.*, 2005). This CXCR4 activation by CXCL12 further stimulated EGF receptor phosphorylation and its downstream kinases, ERK1/2, Akt and c-Src that might link several signallings of cell proliferation in ovarian cancer cells (Porcile *et al.*, 2005). On the other hand, VEGF, a potent angiogenic factor, induced upregulation of *CXCR4* gene expression in vascular endothelial cells, and expression of both VEGF and CXCL12 was very high in ascites of patients with advanced ovarian cancers (Kryczek *et al.*, 2005). The cross-talk of CXCL12/CXCR4 with EGF/EGF receptor and/or VEGF/VEGF receptor might thus provide important signallings for both cell proliferation and angiogenesis in ovarian cancers.

CXCL12/CXCR4 pathway is also expected to be clinically involved in acquirement of malignant characteristics of human ovarian cancers. Of 14 chemokine receptors, only CXCR4 protein was found to be expressed in ovarian cancer cell lines and in ascites from patients with ovarian cancers (Scotton *et al.*, 2001). The CXCL12/CXCR4 pathway has been implicated in

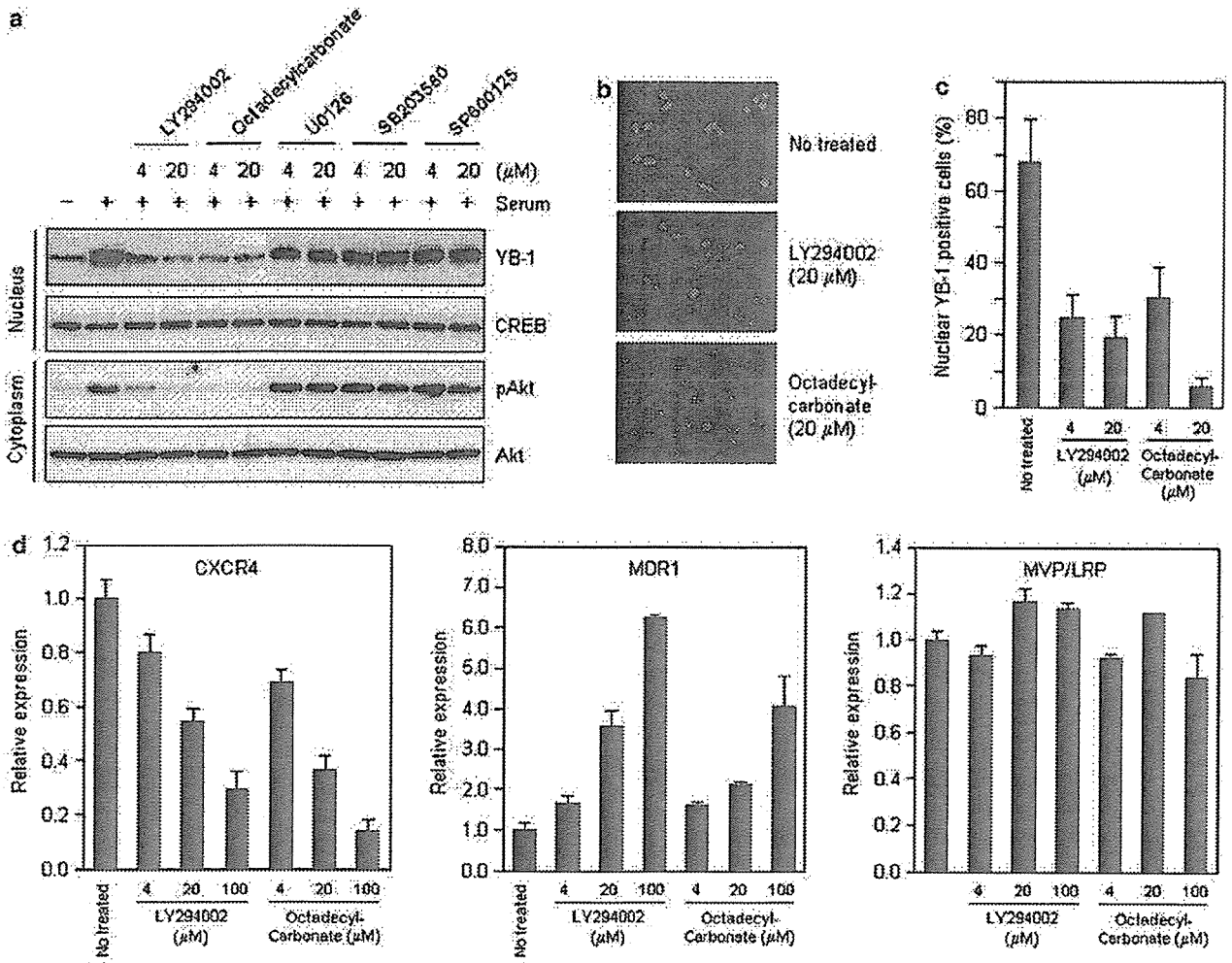

Figure 5 Akt activity is required for YB-1 nuclear accumulation and transcriptional regulation by YB-1. (a) The effect of kinase inhibitors on the nuclear accumulation of YB-1 in SKOV-3 cells. Inhibitors were added 3 h before serum stimulation and nuclear extracts were prepared 1 h after serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed with nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed on cytoplasmic extracts. CREB and Akt are shown as a loading control. (b) Immunofluorescent staining for YB-1. SKOV-3 cells were treated with LY294002 or octadecylcarbonate for 24 h and then stained with YB-1. Cells were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (c) Quantitative analysis of YB-1 nuclear localization in SKOV-3 cells as shown in Figure 2b. Data are mean of three independent experiments; bars ±s.d. (d) QRT-PCR for MDR1, MVP/LRP, CXCR4 and housekeeping gene GAPDH. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH}) - C_t(\text{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ±s.d.

the development of tumor growth, angiogenesis and metastasis not only in ovarian cancer (Scotton *et al.*, 2002) but also in other tumor types including breast cancer (Muller *et al.*, 2001), melanoma (Robledo *et al.*, 2001; Murakami *et al.*, 2002) and prostate cancer (Darash-Yahana *et al.*, 2004). Jiang *et al.* (2006) further demonstrated that CXCR4 expression could be an important prognostic marker for ovarian cancers: the rate of CXCR4 expression in refractory and recurrent group was significantly higher than that in non-recurrent group. Our previous studies showed a significant association of nuclear localization of YB-1 with unfavorable prognosis of patients with ovarian cancers (Kamura *et al.*, 1999; Huang *et al.*, 2004). Clinicopathological analysis whether nuclear expression of YB-1 can be associated with CXCR4 expression or CXCL12 (SDF-1α) in patients with ovarian cancers is now in progress.

Several studies have focused on the role of Akt/PI3K inhibitors as potential tumor suppressor agents. It has been reported that phosphorylation of Akt and mTOR, an Akt substrate, was frequently detected in ovarian cancer (Altomare *et al.*, 2004). In animal model of ovarian cancer, LY294002, a potent inhibitor of Akt activation, could inhibit cancer growth and ascites formation (Hu *et al.*, 2000). Our study also

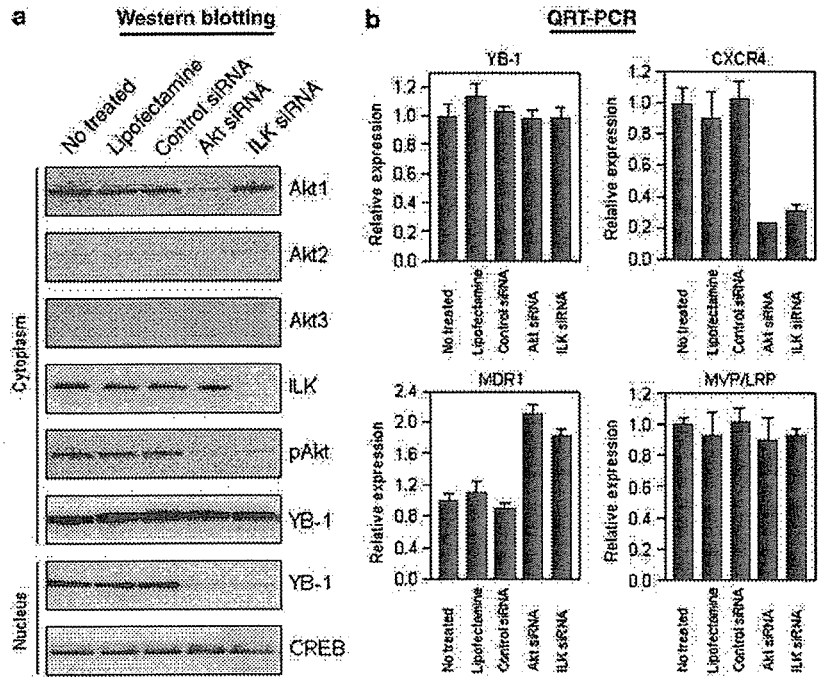

Figure 6 Effect of knock down of Akt and ILK on YB-1 nuclear accumulation, and expression of MDR1, MVP/LRP and CXCR4. (a) SKOV-3 cells were treated with Akt siRNA (100 nM), ILK siRNA (10 nM) or control siRNA (100 nM) for 48 h, and then cytoplasmic and nuclear extracts were prepared. Anti-Akt1, anti-Akt2, anti-Akt3, anti-ILK, anti-pAkt, and anti-YB-1 immunoblots were performed with cytoplasmic extracts, and anti-YB-1 and anti-CREB immunoblots were performed with nuclear extracts. (b) SKOV-3 cells were treated with Akt siRNA (100 nM) or ILK siRNA (10 nM) for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and GAPDH housekeeping gene. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\mathrm{GAPDH}) - C_t(\mathrm{target}))}$ which reflected target genes normalized to GAPDH levels. Data were mean of three independent experiments; bars ±s.d.

demonstrated that both Akt phosphorylation and YB-1 nuclear localization were blocked by administration of LY294002 in SKOV-3 xenograft model. Nuclear localization of YB-1 is induced through various pathways including Akt (see Introduction). The Akt-dependent pathway for YB-1 nuclear localization would provide further insight how Akt-targeting anticancer therapeutic strategy could be developed.

In conclusion, we have identified several genes that are regulated by YB-1 and/or its nuclear localization. Further immunohistochemical analysis should be required to elucidate the role of YB-1 in the expression of *CXCR4* and other relevant genes that are associated with the clinicopathological characteristics in human ovarian cancers. Based on our present experimental results, we aim to present YB-1 and YB-1-dependent gene networks as molecular targets for the further development of novel anticancer therapeutic strategies.

Materials and methods

Cell culture and reagents

OVCAR-3 and SKOV-3 were purchased from American Type Culture Collection (Manassas, VA, USA). RMG-I, RMG-II, RMG-III, RMBG and RTSG were kindly provided by Dr S Nozawa, Department of Obstetrics and Gynecology, Keio University. These cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2. LY294002 and U0126 were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Hu *et al.*, 2000), SB203580 (Cuenda *et al.*, 1995), and SP600125 (Bennett *et al.*, 2001) were obtained from Calbiochem (San Diego, CA, USA). Anti-YB-1 was generated as described previously (Ohga *et al.*, 1996). Anti-CREB, anti-PKB/Akt, anti-phospho-PKB/Akt, anti-ILK, Akt siRNA and ILK siRNA were obtained from Cell Signaling Technology (Beverly, MA, USA).

Western blotting

Western blotting was performed as previously described (Kaneko *et al.*, 2004). Cells were lysed in buffer A (10 mM HEPES (pH7.9), 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% v/v IGEPAL, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml aprotinin and leupeptin) for 10 min on ice, and then centrifuged for 3 min at 15 000 r.p.m. The supernatant fractions (cytoplasmic soluble proteins) were collected. The nuclear pellet was then washed and then lysed in buffer C (20 mM HEPES (pH7.9), 200 mM NaCl, 1 mM EDTA, 5% v/v glycerol, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml aprotinin and leupeptin). Lysates were incubated on ice for 2 h, and then centrifuged 15 000 r.p.m. for 5 min. The lysates were separated by sodium dodecyl sulfate–polyacryl amide gel electrophoresis (SDS–PAGE), and then were transferred to a nitrocellulose membrane. The membrane were incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase (Cell Signaling Technology)

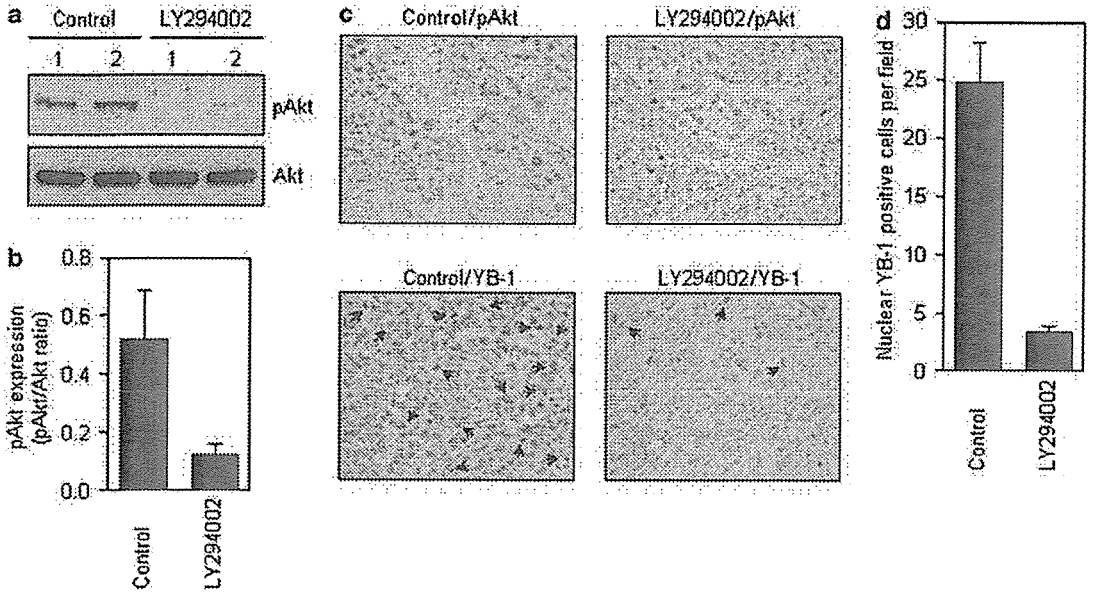

Figure 7 Effect of LY294002 on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft. (a) Effect of LY294002 on Akt phosphorylation in SKOV-3 xenograft. SKOV-3 cells were injected subcutaneously (5.0×10^6 cells/0.1 ml/mouse). When tumors reached approximately 1000–2000 mm^3, animals were randomly assigned to two groups of five. The first group received i.p. injections of DMSO as a control. The second group received i.p. injections of 50 mg/kg LY294002. One hour after LY294002 injection, mice were killed humanely (while anesthetized) by cervical dislocation and tumors were excised. Western blot analysis was carried out using cytosolic extracts prepared from tumor tissues from two animals treated with or without drug. (**b**) Quantitative analysis of Akt phosphorylation in SKOV-3 tumor xenograft. Levels of Akt phosphorylation were normalized to their nonphosphorylated form as shown in Figure 7a. Data are expressed as mean ± s.d. of three to five mice. (**c**) Immunohistochemical staining was carried out using conventional protocols. The arrows indicate positive cell nuclei staining for YB-1 (× 200 magnification). (**d**) Quantitative analysis of YB-1 nuclear localization in SKOV-3 tumor xenograft. YB-1 nuclear localization was determined by counting the number of positive YB-1 nuclear cells in high-power fields as shown in Figure 7b. Data were mean of each section (five sections per mouse). Columns, mean; bars ± s.d.

and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Bands on Western blots were analysed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD, USA).

Oligonucleotide microarray analysis

The siRNA corresponding to nucleotide sequences of the YB-1 (5′-GGU UCC CAC CUU ACU ACA U-3′) was purchased from QIAGEN Inc. (Valencia, CA, USA). A negative control siRNA was obtained from Invitrogen (Carlsbad, CA, USA). siRNA duplexes were transfected using LipofectAMINE2000 and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations. Duplicate samples were prepared for microarray hybridization. At 48 h after siRNA transfection, total RNA was extracted from cell cultures using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan). Total RNA (2 μg) was reverse-transcribed using GeneChip 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA, USA) and then labeled with Cy5 or Cy3. The labeled cRNA was applied to the oligonucleotide microarray (Human Genome U133 Plus 2.0 Array, Affymetrix). The microarray was scanned on a GeneChip Scanner3000 and the image was analysed using a GeneChip Operating Software ver1.

Correlation analysis of gene expression, and clustering of cell lines and genes expression

Gene expression data for the 60 human tumor cell lines were obtained from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), expressed as log of the mRNA levels in cell line/mRNA levels in reference pool in the NCI screen. Pearson correlation coefficients were calculated for each gene–gene pair. Hierarchical clustering can be used to group cell lines and genes in term of their patterns of gene expression. To obtain cluster trees for genes that showed distinct expression patterns across the 60 cell lines, we used the program 'Cluster' and 'Tree View' (http://rana.lbl.gov/) with average linkage clustering and a correlation metric (Eisen *et al.*, 1998).

Quantitative real-time polymerase chain reaction

RNA was reverse transcribed from random hexamers using AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed using the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA, USA) as described previously (Maruyama *et al.*, 2006). In brief, the PCR amplification reaction mixtures (20 μl) contained cDNA, primer pairs, the dual-labeled fluorogenic probe, and TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and at 95°C for 10 min, and then alternating for 40 cycles between 95°C for 15 s and 60°C for 1 min. The primer pairs and the probe were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\mathrm{GAPDH}) - C_t(\mathrm{target}))}$ which reflected target gene expression normalized to GAPDH levels.

Immunofluorescence

Cells were plated on glass coverslips in six-well plates and allowed to attach overnight. Then, cells were rinsed with PBS

and then fixed in 4% paraformaldehyde/PBS for 30 min. Cells were rinsed twice with PBS and then permeabilized with 0.5 ml of solution containing 5% BSA, 0.2% Triton X-100 in PBS for 90 min. After 1 h of blocking with 2% goat serum, the cells were incubated overnight with primary antibody at 4°C in 1% BSA in PBS. Cells were then rinsed three times with PBS and incubated with 1 μg/ml of Alexa Flour 546-labeled secondary antibody (Molecular Probe, Eugene, OR, USA) in 1% BSA in PBS for 60 min. Coverslips were mounted on slide glasses using gel mount and viewed using an Olympus BX51 florescence microscope (Tokyo, Japan) and photographed with Olympus DP-70 digital camera.

Tumor xenograft study

Male BALB/c nude mice were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). SKOV-3 cells were harvested and resuspended in PBS. The suspension was injected subcutaneously in the mice (5.0×10^6 cells/0.1 ml/mouse). When tumors reached about 1000–2000 mm^3, animals were randomly assigned to two groups of five mice each. The first group received i.p. injections of DMSO as control. The second group received i.p. injection of LY294002 at 50 mg/kg. At 1 h after LY294002 injection, mice were killed humanly (mice still anesthetized) by cervical dislocation and tumors were excised. For immunohistchemistry, one part of the tumor tissue was fixed in formalin and embed in paraffin.

Acknowledgements

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EGFR mutation in various tissues

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Abstract Somatic mutations have been demonstrated in various tumors. *EGFR* mutations were first demonstrated in adenocarcinoma of the lung, and a large-scale retrospective study has clearly shown that these mutations are specifically observed in this form of the disease. Recently, possible occurrence of *EGFR* mutations in other tumor types including ovarian and colorectal malignancies has been reported. This raises the possibility of application of EGFR-specific tyrosine kinase inhibitors (EGFR-TKI) to the treatment of these malignancies, although broad success in this venture would depend on the frequency of such mutations. In this article, we discuss somatic mutations in various tumors as well as potential application of TKI to their treatment. Ethnic difference in the frequency of somatic mutations is another area of interest since it is closely related to clinical response to EGFR-TKIs. Preliminary studies have revealed such ethnic variations regarding *EGFR* mutation and gene amplification. Ethnic difference of transcriptional regulation of *EGFR* has also been demonstrated. We recently found a biomarker related to clinical response to EGFR-TKI that might explain the ethnic differences in response to this therapy. Various tyrosine kinases are known targets of TKIs. Thus genomics of individual patients may allow personalized target-based therapeutics.

Keywords EGFR mutation · Tyrosine kinase inhibitor · Ethnicity · HLA

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EGFR mutation in various cancers

Somatic mutations have been demonstrated in various tumors. *EGFR* mutations were first demonstrated in adenocarcinoma of the lung, and a large-scale retrospective study has clearly shown that these mutations are specifically observed in this form of the disease [10]. However, extensive analysis of somatic mutation in various tumors subsequently demonstrated the existence of *EGFR* somatic mutation in many human tumors such as colorectal and head and neck cancer, renal cell carcinoma, prostate cancer, and cholangiocarcinoma [4, 7, 8]. Gwak et al. [5] reported *EGFR* mutation in cholangiocarcinoma and found that it was detectable in 13.6% (3/22) of patients. The type of mutation was deletion of exon 19. This is commonly observed in intrahepatic and poorly differentiated tumors. These and other researchers also reported this *EGFR* mutation in squamous cell head and neck carcinoma [7], and Cohen's group demonstrated a new mutation on *erb2* and gene amplification in this disease [3]. The mutation has also been reported in persistent ovarian and primary peritoneal carcinoma in clinical phase II trials of gefitinib [14]. Similar types of mutation have been reported in lung cancers, although these seem to be of minor occurrence [4]. Thus somatic mutations of *EGFR* exist in various tumors. Because of limited samples, it